Received: 13 January 2023 | Revised: 17 August 2023 | Accepted: 18 August 2023

https://doi.org/10.1016/j.rpth.2023.102204

rpth research & practice in thrombosis & haemostasis

ORIGINAL ARTICLE

Angiopoietin-2 binds to multiple interactive sites within von Willebrand factor

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Handling Editor: Prof Cihan Ay

Abstract

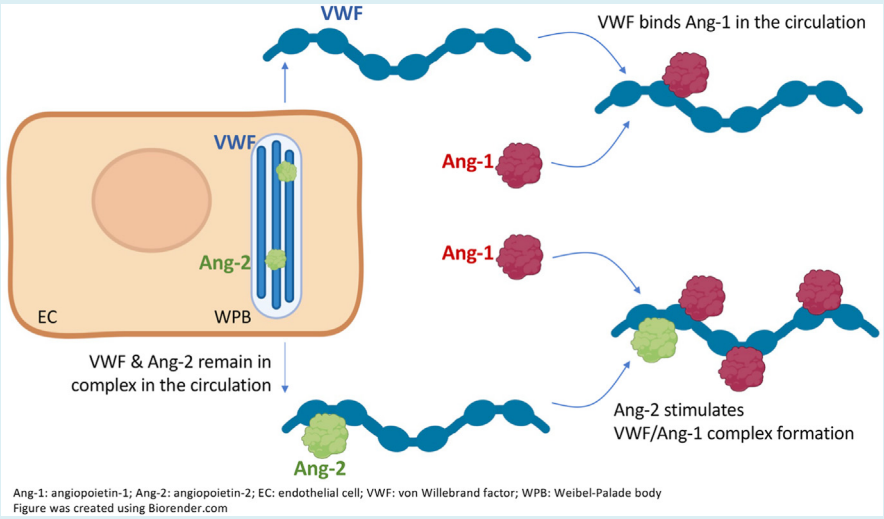
Background: Biosynthesis of von Willebrand factor (VWF) in endothelial cells drives the formation of storage-organelles known as Weibel-Palade bodies (WPBs). WPBs also contain several other proteins, including angiopoietin-2 (Ang-2).

Objectives: At present, the molecular basis of the VWF–Ang-2 interaction is poorly understood. Here, we used immunosorbent-binding assays and specific recombinant VWF fragments to analyze VWF–Ang-2 interactions.

Results: We found that VWF bound to immobilized Ang-2 most efficiently (half-maximal binding at 0.5 ± 0.1 μg/mL) under conditions of high $CaCl_2$ (10 mM) and slightly acidic pH (6.4-7.0). Interestingly, several isolated recombinant VWF domains (A1/Fc, A2/Fc, D4/Fc, and D'D3-HPC4) displayed dose-dependent binding to immobilized Ang-2. Binding appeared specific, as antibodies against D'D3, A1, and A2 significantly reduced the binding of these domains to Ang-2. Complexes between VWF and Ang-2 in plasma could be detected by immunoprecipitation- and immunosorbent assays. Unexpectedly, control experiments also revealed complexes between VWF and angiopoietin-1 (Ang-1), a protein structurally homologous to Ang-2. Furthermore, direct binding studies showed dose-dependent binding of VWF to immobilized Ang-1 (half-maximal binding at 1.8 ± 1.0 μg/mL). Interestingly, rather than competing for Ang-1 binding, Ang-2 enhanced the binding of VWF to Ang-1 about 3-fold. Competition experiments further revealed that binding to VWF does not prevent Ang-1 and Ang-2 from binding to Tie-2.

Conclusion: Our data show that both Ang-1 and Ang-2 bind to VWF, seemingly using different interactive sites. Ang-2 modulates the binding of VWF to Ang-1, the (patho)-physiological consequences of which remain to be investigated.

Stéphanie Roullet and Olivier D. Christophe contributed equally to this study.



KEYWORDS
angiopoietin-1, angiopoietin-2, endothelial cells, von Willebrand factor, Weibel-Palade bodies

Essentials

- Von Willebrand factor (VWF) and angiopoietin-2 (Ang-2) colocalize in Weibel-Palade bodies.
- Ang-2 and VWF circulate in complex, and VWF contains multiple binding sites for Ang-2.
- VWF also binds angiopoietin-1 and this interaction is stimulated by the presence of Ang-2.
- The (patho)-physiological consequences of these interactions remain to be determined.

1 | INTRODUCTION

von Willebrand factor (VWF) is a large multifunctional multimeric protein that plays a crucial role in the recruitment of platelets to the sites of injury, and functions as a carrier-protein for coagulation factor (F) VIII (FVIII) in the circulation [1]. When synthesized in endothelial cells, VWF is the driving force behind the biogenesis of Weibel-Palade bodies (WPBs) [2]. WPBs are large rod-shaped secretory organelles unique to endothelial cells, which mediate the acute secretion of proteins in response to external signals [3]. These organelles are the residence of a variety of proteins with diverse biological functions [1,4].

Fiedler et al. [5,6] have shown that the Tie-2 ligand angiopoietin-2 (Ang-2) is colocalized with VWF in WPBs of endothelial cells. Ang-2 is expressed weakly by the resting endothelium, but its expression is strongly up-regulated following endothelial activation [7–10].

Besides their spatial co-localization in WP-bodies, Ang-2 and VWF also share a functional connection to angiogenic processes. It has been demonstrated that the binding of Ang-2 to Tie-2 destabilizes the resting endothelium, thereby promoting vascular endothelial growth factor-induced vessel sprouting [11,12]. In contrast to Ang-2, VWF displays an anti-angiogenic role by a mechanism that is not yet fully understood [13,14].

The spatial and functional relationship between VWF and Ang-2 has prompted studies to their potential interaction. Indeed, Mobayen et al. [15] recently demonstrated that both proteins remain associated in solution upon stimulated release from cultured endothelial cells. Further experiments showed an important role of the VWF A1-domain in binding Ang-2. Interestingly, complex formation between VWF and Ang-2 did not affect the binding of Ang-2 to Tie-2 nor was the binding of VWF to platelets affected [15].

The notion that VWF and Ang-2 remain associated upon secretion from cultured endothelial cells raises the question whether VWF/Ang-2 complexes can be found in the circulation? And if so, could it be possible that VWF is also capable of binding to Angiopoietin-1 (Ang-1), which displays structural homology to Ang-2 [16]? In the present study, we further analyzed the binding of VWF to Ang-2, identifying multiple binding site for Ang-2. In addition, we were able to detect VWF/Ang-2 complexes in plasma using immunoprecipitation- and immunosorbent assays. Unexpectedly, we also detected circulating complexes of VWF with Ang-1, the interaction of which was enhanced by the addition of Ang-2.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All volunteers and patients provided informed written consent according to the Declaration of Helsinki. All protocols were approved by the local

review and ethics committees. Plasmas were collected from patients with hemophilia A at the hemophilia treatment center at the University Hospital of Nantes. All plasma samples were from white males.

2.2 | Proteins

Recombinant human angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), Tie-2/Fc, and ADAMTS-13 were purchased from R&D Systems. Plasma-derived VWF was purified from VWF concentrates as described [17]. Purified VWF was labeled with ^{125}I (Perkin-Elmer) using IodoGen (Pierce Chemical Co) as described [18]. Specific radioactivity varied from 3 to 6 µCi/µg. Degraded VWF (VWF-degr) was prepared as described [19]. Recombinant VWF fragments D'-D3-HPC4, A1/Fc, A2/Fc, A3/Fc, and D4/Fc have been described previously [17,20].

2.3 | Antibodies

Rabbit polyclonal anti-human VWF antibodies were from Dako (Dako France). Murine monoclonal antibodies Mab723, Mab418, and Mab487 have been described previously [21–23]. Monoclonal antibodies Mab0983 (anti-human Ang-2), Mab923 (anti-Ang-1), and Mab22641 (anti-VWF A2 domain) were from R&D systems. Peroxidase-labeled monoclonal mouse anti-human Fc antibody was from Southern Biotech. Mouse monoclonal antibody HPC4 was from Roche. Peroxidase-labeled polyclonal goat anti-mouse immunoglobulin G (IgG) antibodies were from Sigma.

2.4 | Binding of VWF to Ang-2 under static conditions

Binding of VWF to Ang-2 was assayed as previously described for VWF binding to osteoprotegerin [24]. In brief, Ang-2 (1 µg/mL) was coated in carbonate buffer (pH 9.6) on 96-well microtiter plates (Greiner Merck Eurolab). Wells were washed thrice with Bis-Tris-buffer (20 mM Bis-Tris buffer, pH 6.8; 0.1% Tween-20; 0.03% bovine serum albumin [BSA]). Nonspecific binding sites were blocked with 3% BSA (1 hour, 37 °C). Then, serial dilutions of VWF or derivatives thereof were added to the wells and incubated at 25 °C for 1 hour. Different conditions were used, which are depicted in Figure 1: Bis-Tris buffer dilutions of VWF derivatives in 20 mM Bis-Tris buffer (pH 6.8) containing 0 to 40 mM $CaCl_2$; 20 mM Bis-Tris buffer (pH 6.8) + 10 mM $CaCl_2$ containing 0 to 100 mM NaCl, KCl, $NaCH_3CO_2$, or KCH_3CO_2; 20 mM Bis-Tris buffer + 10 mM $CaCl_2$ with various pH values (5.8 to 7.2). For Figures 2 and 3, the following buffer composition was used: 20 mM Bis-Tris buffer (pH 6.8) + 10 mM $CaCl_2$. Bound VWF was probed using horseradish peroxidase-labeled polyclonal anti-VWF antibodies. Binding of Ang-2 to immobilized VWF was analyzed using similar buffer conditions (20 mM Bis-Tris buffer [pH 6.8] + 10 mM $CaCl_2$, 0.1% Tween-20, 0.03% BSA). Bound Ang-2 was probed with peroxidase-labeled anti-mouse IgG antibodies. VWF domains fused to Fc were probed using peroxidase-labeled anti-Fc antibodies, while D'-D3-HPC4 was probed using peroxidase-labeled antibody HPC4. Binding of VWF to immobilized ADAMTS-13 was performed as described [25]. Binding of VWF to immobilized Ang-1 was performed in 20 mM Hepes pH 7.0, 2.5 mM $CaCl_2$, 100 mM NaCl, 0.5% BSA, 0.1% Tween. Binding of Tie-2/Fc to immobilized Ang-1 or Ang-2 was performed in 20 mM Hepes pH 7.0, 2.5 mM $CaCl_2$, 100 mM NaCl, 0,5% BSA, 0,1% Tween. Bound Tie-2/Fc was probed using peroxidase-labeled anti-human Fc antibodies. Detection of peroxidase-bound antibodies was performed as indicated via hydrolysis of O-phenylenediamine or 3,3',5,5'-tetramethylbenzidine.

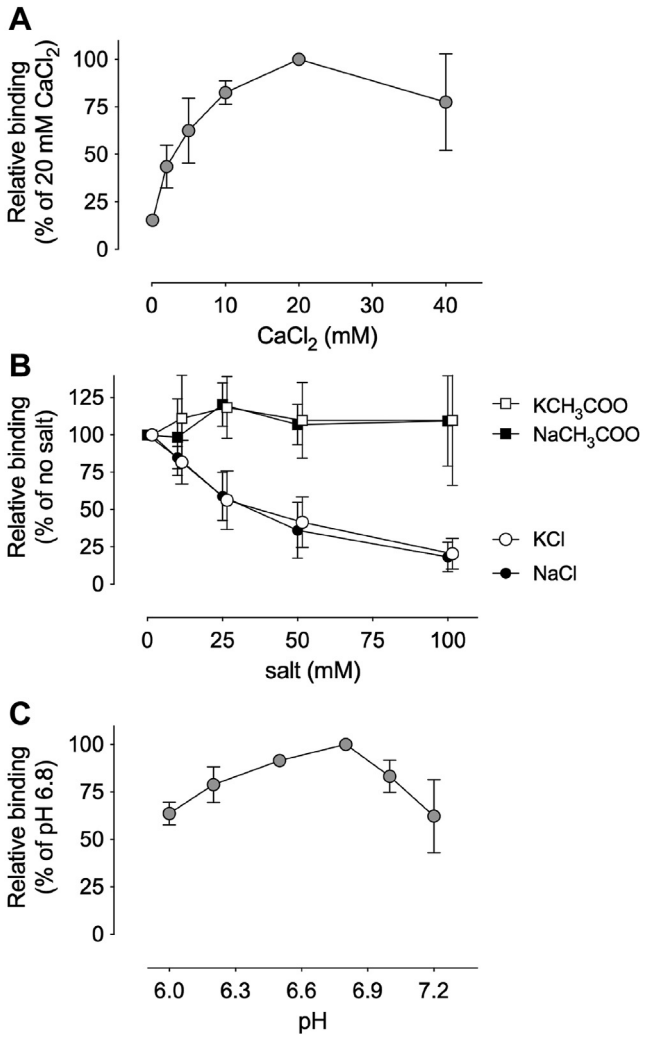


FIGURE 1 Biochemical parameters determining binding of VWF to Ang-2.VWF (2.5 µg/mL) was added to immobilized Ang-2 (1 µg/mL) in 20 mM Bis-Tris buffer pH 6.8, 0.1% Tween-20, 0.03% bovine serum albumin (Bis-Tris-buffer), with different additions: (A) Bis-Tris-buffer + 0-40 mM $CaCl_2$; (B): Bis-Tris-buffer + 10 mM $CaCl_2$ with 0- 100 mM NaCl (closed circles), KCl (open circles), $NaCH_3CO_2$ (closed squares), or KCH_3CO_2 (open squares); (C) Bis-Tris-buffer + 10 mM $CaCl_2$ with pH varying between 6.0 and 7.2. Bound VWF was probed using peroxidase-labeled polyclonal anti-VWF antibodies and detected via hydrolysis of O-phenylenediamine. Data represent mean values of 3 experiments ± SD. Ang-2, angiopoietin-2; VWF, von Willebrand factor.

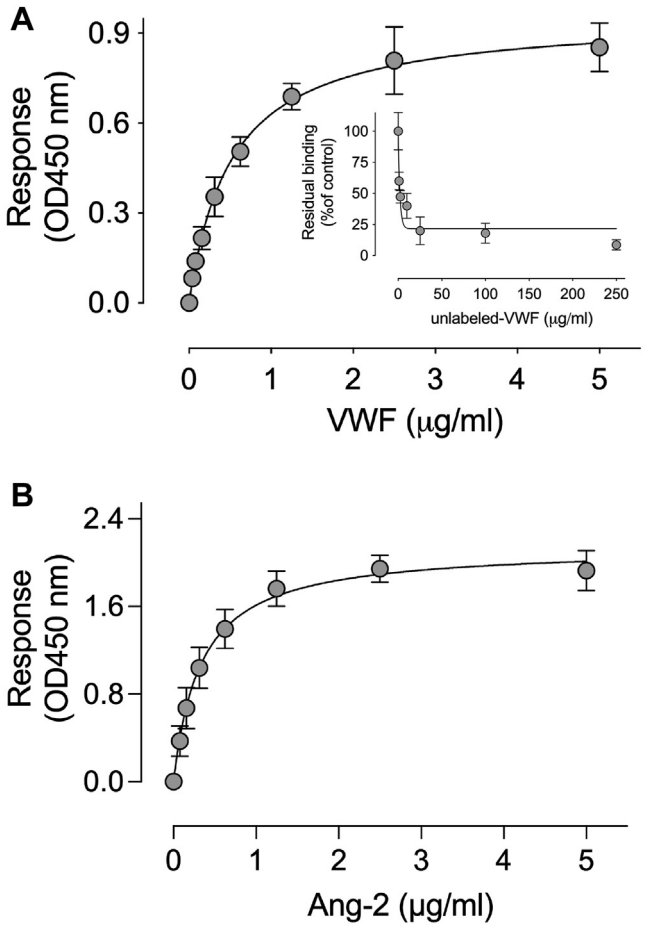


FIGURE 2 Reciprocal binding between VWF and Ang-2.(A) Purified VWF (0-5 μg/mL) was added to immobilized Ang-2 (1 μg/mL) in 20 mM Bis-Tris buffer pH 6.8, 10 mM $CaCl_2$, 0.1% Tween-20, 0.03% bovine serum albumin. Bound VWF was probed using peroxidase-labeled polyclonal anti-VWF antibodies and detected via hydrolysis of 3,3',5,5'-tetramethydlbenzidine. *inset:* I^{125}-labeled VWF (2.5 μg/mL) was incubated in the absence or presence of non-labeled purified VWF (0-250 μg/mL) with immobilized Ang-2 (1 μg/mL). After washing thrice, wells were analyzed for residual radioactivity. Plotted is the residual radioactivity (percentage of I^{125}-labeled VWF in the absence of unlabeled VWF) vs concentration of unlabeled VWF. (B) Recombinant Ang-2 (0-5 μg/mL) was added to immobilized VWF (5 μg/mL) in 20 mM Bis-Tris buffer pH 6.8, 10 mM $CaCl_2$, 0.1% Tween-20, 0.03% bovine serum albumin. Bound Ang-2 was probed using anti-Ang-2 and peroxidase-labeled anti-mouse Fc antibodies, with detection via hydrolysis of 3,3',5,5'-tetramethydlbenzidine. Data represent mean values of 3 experiments ± SD. Ang-2, angiopoietin-2; VWF, von Willebrand factor.

2.5 | Inhibition and competition assays

Binding of I^{125}-labeled VWF (2.5 μg/mL) to immobilized Ang-2 (1 μg/mL) was assessed in 20 mM Bis-Tris buffer pH 6.8, 50 mM $CH_3COOHNa$, 10 mM $CaCl_2$, 0.1% Tween, 0.03% BSA in the absence or presence of unlabeled purified VWF (0-250 μg/mL). After washing the wells thrice with the same buffer, isolated wells were analyzed for the presence of residual radioactivity. For the recombinant VWF domains, a fixed concentration (2.5 μg/mL for A1/Fc and A2/Fc and 5 μg/mL for D4/Fc and D'D3-HPC4) was preincubated in the absence or presence of a 20-fold molar excess of antibody for 30 minutes before addition to Ang-2 coated wells (1 μg/mL). For inhibition by FVIII, concentrations of 0 to 30 μg FVIII/mL were used. For ADAMTS-13, VWF (5 μg/mL) was preincubated with Ang-2 (0-25 μg/mL) in 20 mM Bis-Tris pH 6.8, 50 mM $CH_3COOHNa$, 10 mM $CaCl_2$, 0.1% Tween, 0.03% BSA for 30 minutes, and then diluted 5-fold in 20 mM Hepes pH 7.0, 2.5 mM $CaCl_2$, 100 mM NaCl, 0.5% BSA, 0.1% Tween before addition to ADAMTS-13-coated wells.

2.6 | Immunoprecipitation and western blotting

Dynabeads protein G (Dynal) were washed thrice with washing buffer (0.1 M Na-Acetate; pH 5.0) prior to the addition of purified immunoglobulins, ie, either Mab0983 directed against human Ang-2 or MAb487 directed against human VWF and the relevant non-binding controls. These mixtures were incubated for 90 minutes at room temperature. Beads were collected using a magnetic particle collector (MPC-1, Dynal), and washed thrice with 0.1 M Na-Acetate (pH 5.0) or 0.1% Tween and subsequently with phosphate-buffered saline. Following incubation with human normal pool plasma (1 mL; Cryopep, Maugio, France) for 2 hours at 4 °C, beads were washed thrice with phosphate-buffered saline. Subsequently, beads were boiled in reducing buffer and analyzed via 4% to 12% (w/v) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Invitrogen). After electrophoresis, proteins were transferred to polyvinyl-difluoroacetate membranes (Millipore) and incubated with either monoclonal anti-human Ang-2 antibody Mab0983 or peroxidase-labeled rabbit polyclonal anti-human VWF antibodies. Mab0983 was subsequently detected with peroxidase-labeled goat anti-mouse IgG antibodies (TebuBio). Immunoprecipitations using an isotype-matched control (mouse and goat IgG from R&D) were used as negatives control.

2.7 | Immunosorbent assays

Wells coated with anti-Ang-1 or anti-Ang-2 antibodies were incubated with plasma (diluted 2-fold or 5-fold) for 1 hour at 37 °C. Bound complexes of VWF/Ang-1 or VWF/Ang-2 were probed using peroxidase-labeled polyclonal anti-VWF antibodies and detected via hydrolysis of 3,3',5,5'-tetramethylbenzidine.

3 | RESULTS

3.1 | Biochemical parameters required for the complex formation between VWF and Ang-2

To explore optimal conditions for VWF and Ang-2 to interact, we first determined how their interaction was affected by $CaCl_2$. As starting-

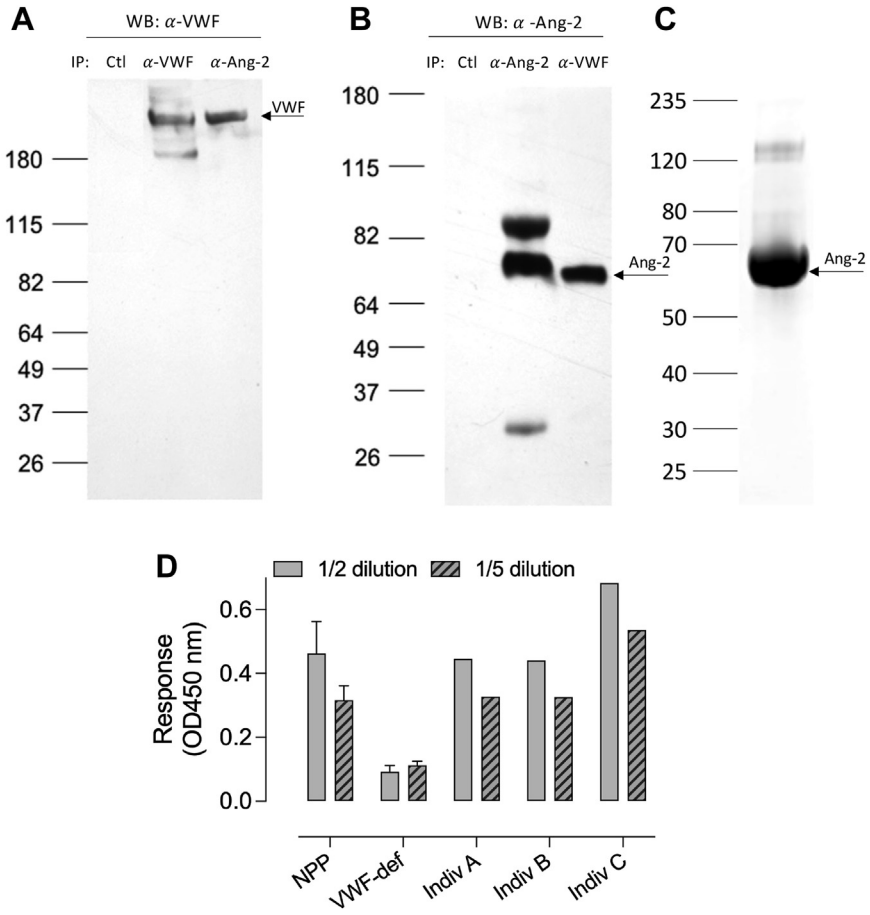


FIGURE 3 Circulating complexes of VWF and Ang-2.Normal plasma was incubated with Dynabeads Protein G containing isotype-matched control IgG, anti-VWF, or anti-Ang-2 antibodies. Eluates were subjected to western blotting using antibodies against VWF (A) or Ang-2 (B). (B) the Ang-2 band was identified by its migration at a molecular weight of 69 kDa, which is similar to that of recombinant Ang-2 (C), which migrates at a molecular weight of 68 kDa. (D) Microtiter wells coated with anti-Ang-2 antibodies were incubated with diluted plasma (gray bars: 2-times diluted; hatched bars: 5-times diluted). Bound complex was then probed using peroxidase-labeled polyclonal anti-VWF antibodies and detected via hydrolysis of 3,3',5,5'-tetramethylbenzidine. Data represent mean values of 3 experiments ± SD for NPP and VWF-deficient plasma, and the mean of a duplicate measurement for the individual plasmas. Ang-2, angiopoietin-2; Indiv A-C, plasma of individual A, B, and C; NPP, normal pooled plasma; VWF-def, VWF-deficient plasma.

buffer, we used 20 mM Bis-Tris (pH 6.8)/0.1% Tween-20/0.03% BSA), to which different concentrations of $CaCl_2$ were added. VWF binding to immobilized Ang-2 proved to be strongly $CaCl_2$-dependent, and maximum binding was reached at 10 to 30 mM $CaCl_2$ (Figure 1A). In further experiments, $CaCl_2$ concentration was kept at 10 mM. In a next series of experiments, the effect of ionic strength was evaluated. Binding was assessed 20 mM Bis-Tris (pH 6.8)/0.1% Tween-20/0.03% BSA/10 mM $CaCl_2$, with the addition of various concentrations of NaCl, KCl, $NaCH_3CO_2$, or KCH_3CO_2. VWF binding to Ang-2 decreased with increasing NaCl or KCl concentrations to reach <50% binding in the presence of 40 mM salt, and <20% binding in the presence of 100 mM salt (Figure 1B). In contrast, no inhibition was observed with $NaCH_3CO_2$ or KCH_3CO_2 up to concentrations of 100 mM (Figure 1B). These data indicate that Cl^- concentrations exceeding 40 mM strongly reduce binding of VWF to Ang-2, and neither NaCl nor KCl was added to the binding buffer in subsequent experiments, unless specifically indicated. Finally, the interaction of VWF with Ang-2 was evaluated as a function of pH. VWF binding to Ang-2 was maximal at pH values between 6.4 and 7.0 (Figure 1C). Control experiments were performed to ensure that the amount of immobilized Ang-2 remained similar between the different conditions (Supplementary Figure S1). Altogether, optimal binding of VWF to Ang-2 involves high $CaCl_2$ and low Cl^- concentrations at a slightly acidic pH.

3.2 | Dose-dependent interactions between VWF and Ang-2

By using these conditions (20 mM Bis-Tris (pH 6.8)/10 mM $CaCl_2$/0.1% Tween-20/0.03% BSA), an efficient saturable and dose-dependent binding of VWF to Ang-2 was observed (Figure 2A), with half-maximal binding being 0.5 ± 0.1 μg/mL. Binding was specific, as unlabeled VWF efficiently interfered with the binding of ^{125}I-labeled VWF (Inset Figure 2A). Half-maximal inhibition was obtained at a concentration of 1.2 μg/mL (95% CI: 0.6-2.4 μg/mL). In complementary assays, Ang-2 bound in a dose-dependent manner to immobilized VWF, with half-maximal binding being 0.3 ± 0.1 μg/mL (Figure 2B), In summary, our data show that Ang-2 can bind both soluble and immobilized VWF.

3.3 | VWF and Ang-2 circulate as a complex in plasma

Given the finding that VWF and Ang-2 remain associated upon secretion from cultured endothelial cells [15], we next investigated whether this complex can be found in plasma. In first instance, we performed reciprocal co-immunoprecipitation experiments using normal pooled plasma (NPP). For immunoprecipitation experiments,

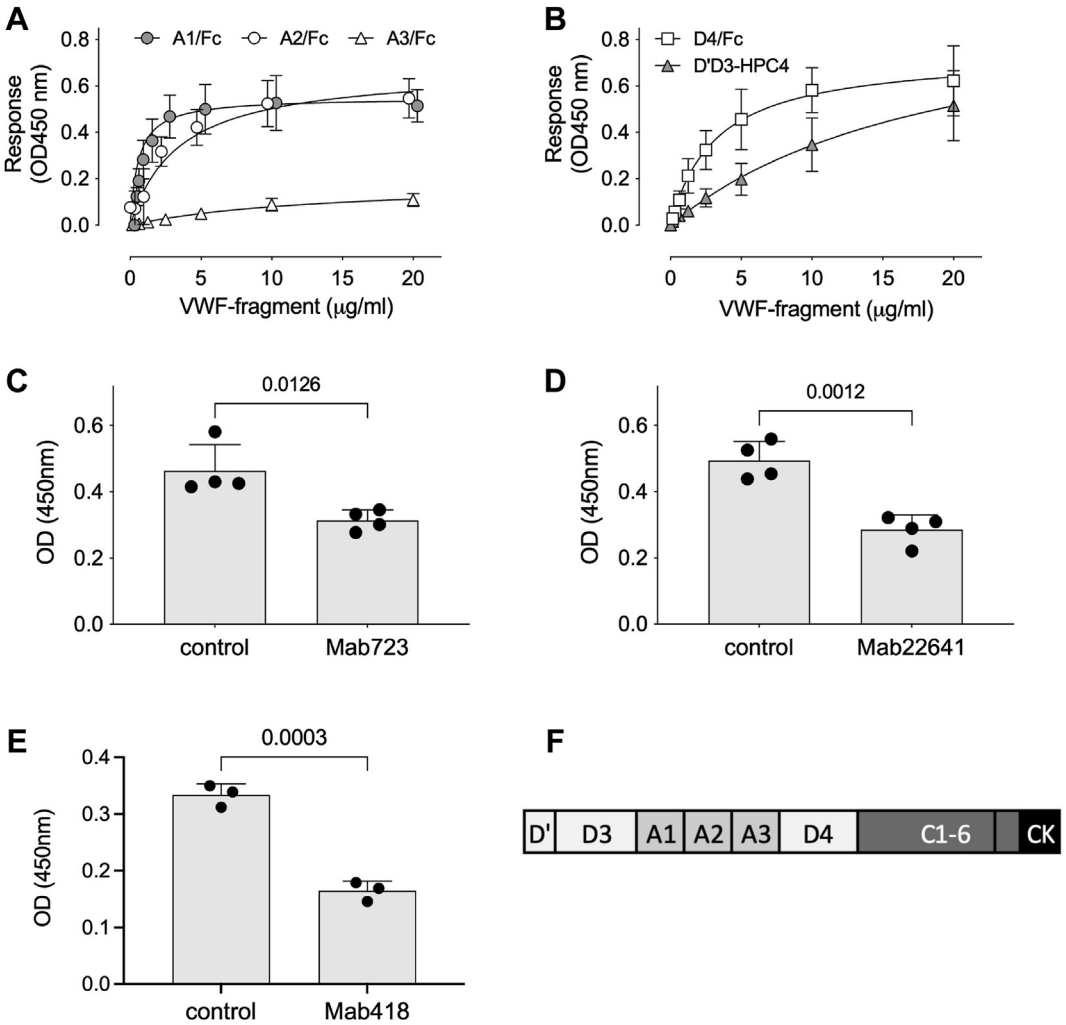


FIGURE 4 Binding of VWF domains to Ang-2. (A) Purified recombinant A-domains fused to Fc (0-20 μg/mL) were incubated with immobilized Ang-2 (1 μg/mL) in 20 mM Bis-Tris buffer pH 6.8, 10 mM $CaCl_2$, 0.1% Tween-20, 0.03% bovine serum albumin. Gray circles represent A1/Fc, open circles represent A2/Fc and open triangles represent A3/Fc. (B) Purified recombinant D4/Fc (open squares) or monomeric D'D3-HPC4 (gray triangles), both 0-20 μg/mL, were incubated with immobilized Ang-2 (1 μg/mL) in 20 mM Bis-Tris buffer pH 6.8, 10 mM $CaCl_2$, 0.1% Tween-20, 0.03% bovine serum albumin. Bound Fc-fragments were probed with peroxidase-labeled anti-Fc antibodies, while D'D3-HPC4 was probed using peroxidase-labeled antibody HPC4. Detection was performed via hydrolysis of 3,3',5,5'-tetramethylbenzidine. (C–E) A1/Fc (2.5 μg/mL), A2/Fc (2.5 μg/mL), and D'D3-HPC4 (5 μg/mL) were incubated in the absence or presence of 20-fold molar excess of antibody Mab723, Mab22641, or Mab418, respectively. After 30-minute incubation, mixtures were added to Ang-2 coated wells (1 μg/mL). Bound A1/Fc, A2/Fc, and D'D3-HPC4 were probed and detected as described in panels (A) and (B). For all panels, data represent mean ± SD of 3 to 4 experiments. Ang-2, angiopoietin-2; VWF, von Willebrand factor.

we used monoclonal antibodies directed against VWF (antibody Mab487; indicated by α-VWF) and Ang-2 (Mab0983; indicated by α-Ang-2). Subsequently, western blotting of these immunoprecipitates allowed the identification of VWF (Figure 3A) or Ang-2 (Figure 3B). Whereas no VWF or Ang-2 could be precipitated using control antibodies, immunoprecipitation with both Ang-2 and VWF antibodies allowed for the detection of VWF (Figure 3A). In line with these results, we also detected the presence of Ang-2 after immunoprecipitation with either antibody (Figure 3B). Since Ang-2 migrates at a different molecular weight (69 kDa) then would be assumed from its amino acid composition (57 kDa), a control with recombinant Ang-2 (migrating at 68 kDa) was included for validation (Figure 3C). We next tested whether VWF/Ang-2 complexes could be detected in individual plasma samples using immunosorbent assays. Wells coated with anti-Ang-2 antibodies were incubated with diluted plasma, and bound complexes were then probed using anti-VWF antibodies. In NPP, we could indeed detect the presence of VWF/Ang-2 complexes using this approach, contrary to the use of VWF-deficient plasma (Figure 3D). VWF/Ang-2 complexes were also detected in the plasma of 3 individuals (Figure 3D). Together, these data show that VWF and Ang-2 are physically associated in plasma, and these complexes seem to be common between individuals.

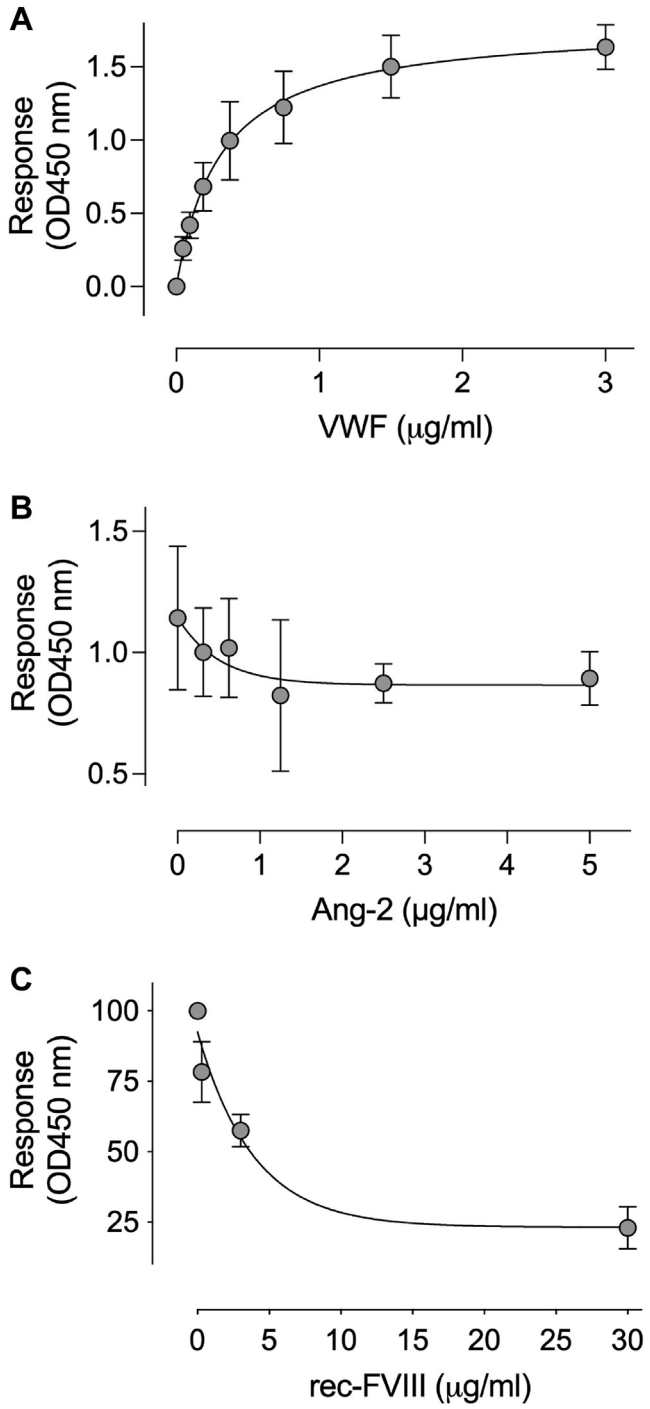


FIGURE 5 Ang-2 vs other binding partners for VWF.(A) Purified VWF (0-3 µg/mL) was added to immobilized recombinant ADAMTS-13 (2.5 µg/mL) in 20 mM Hepes pH 7.0, 2.5 mM $CaCl_2$, 100 mM NaCl, 0.5% BSA, 0.1% Tween-20. (B) VWF (5 µg/mL) was preincubated with Ang-2 (0-25 µg/mL) in 20 mM Bis-Tris buffer pH 6.8, 10 mM $CaCl_2$, 0.1% Tween-20, 0.03% BSA. Subsequently, preparations were diluted 5-fold into 20 mM HEPES (pH 7.0), 100 mM NaCl, 2.5 mM $CaCl_2$, 0.1 % BSA, 0.05% Tween-20 before addition to wells coated with recombinant ADAMTS-13 (2.5 µg/mL). (A, B) Bound VWF was probed using peroxidase-labeled polyclonal anti-VWF antibodies and detected via hydrolysis of 3,3',5,5'-tetramethydlbenzidine. (C) D'D3-HPC4 (5 µg/mL) was incubated in the absence or presence of recombinant FVIII (0-30 µg/mL) for 30 minutes before addition to immobilized Ang-2 (1 µg/mL). Presented is residual D'D3-HPC4 binding (percentage of binding in the absence of FVIII) vs recombinant FVIII. Data represent mean ± SD of 3 to 6 experiments. Ang-2, angiopoietin-2; BSA, bovine serum albumin; VWF, von Willebrand factor.

3.4 | Localization of the Ang-2 binding site on VWF

To identify regions involved in the interaction between VWF and Ang-2, we tested a series of well-defined recombinant VWF fragments for binding to immobilized Ang-2 using similar binding conditions as described for Figure 2A. First, binding of A1/Fc, A2/Fc, and A3/Fc was tested. Whereas no binding of A3/Fc was observed, both A1/Fc and A2/Fc displayed a dose-dependent and saturable association to Ang-2 (Figure 4A). Half-maximal binding was calculated to be 0.6 ± 0.1 µg/mL and 3.4 ± 0.9 µg/mL for A1/Fc and A2/Fc, respectively. Second, binding of D'D3-HPC4 and D4/Fc was analyzed. For both variants, a saturable and dose-dependent binding was observed (half-maximal binding 3.2 ± 0.8 µg/mL for D4/Fc and 19.6 ± 8.5 µg/mL for D'-D3-HPC4; Figure 4B). These data point to at least 4 distinct interactive sites for Ang-2 within the VWF molecule, located in the D'-D3, A1, A2, and D4 domains.

3.5 | Specificity of VWF-domain binding to Ang-2

To address the specificity of the interaction between the various VWF domains and Ang-2, a panel of different monoclonal anti-VWF antibodies were tested for their capacity to interfere with this interaction. With regard to the D4-domain, 3 different monoclonal antibodies were tested. However, none of these interfered with the binding of the D4/Fc to Ang-2. In contrast, for each of the other domains (A1/Fc, A2/Fc, and D'D3-HPC4), binding was reduced in the presence of a monoclonal antibody specific to this domain. Binding was reduced by 30%, 46%, and 51% for A1/Fc, A2/Fc, and D'D3-HPC4, respectively, using monoclonal antibodies Mab723, Mab22641, and Mab418, respectively (Figure 4C–E). Thus, the interactive sites of Ang-2 overlap with binding sites for domain-specific antibodies.

3.6 | VWF–Ang-2 interactions in perspective of FVIII and ADAMTS-13

Ang-2 was shown not to affect platelet-binding to the A1 domain [15]. Here, we wanted to investigate whether Ang-2 binding sites overlap with those of ADAMTS-13 and/or FVIII. VWF displays efficient dose-dependent binding to immobilized recombinant ADAMTS-13 (Figure 5A), compatible with VWF containing binding sites for ADAMTS-13 in the A2 domain and the D4-CK region [26]. The addition of increasing concentrations of Ang-2 affected VWF binding to ADAMTS-13 to a minor extent, which did not reach statistical significance (Figure 5B). As for the D'D3-region, it is known that Mab418 blocks binding of VWF to FVIII. We therefore tested whether

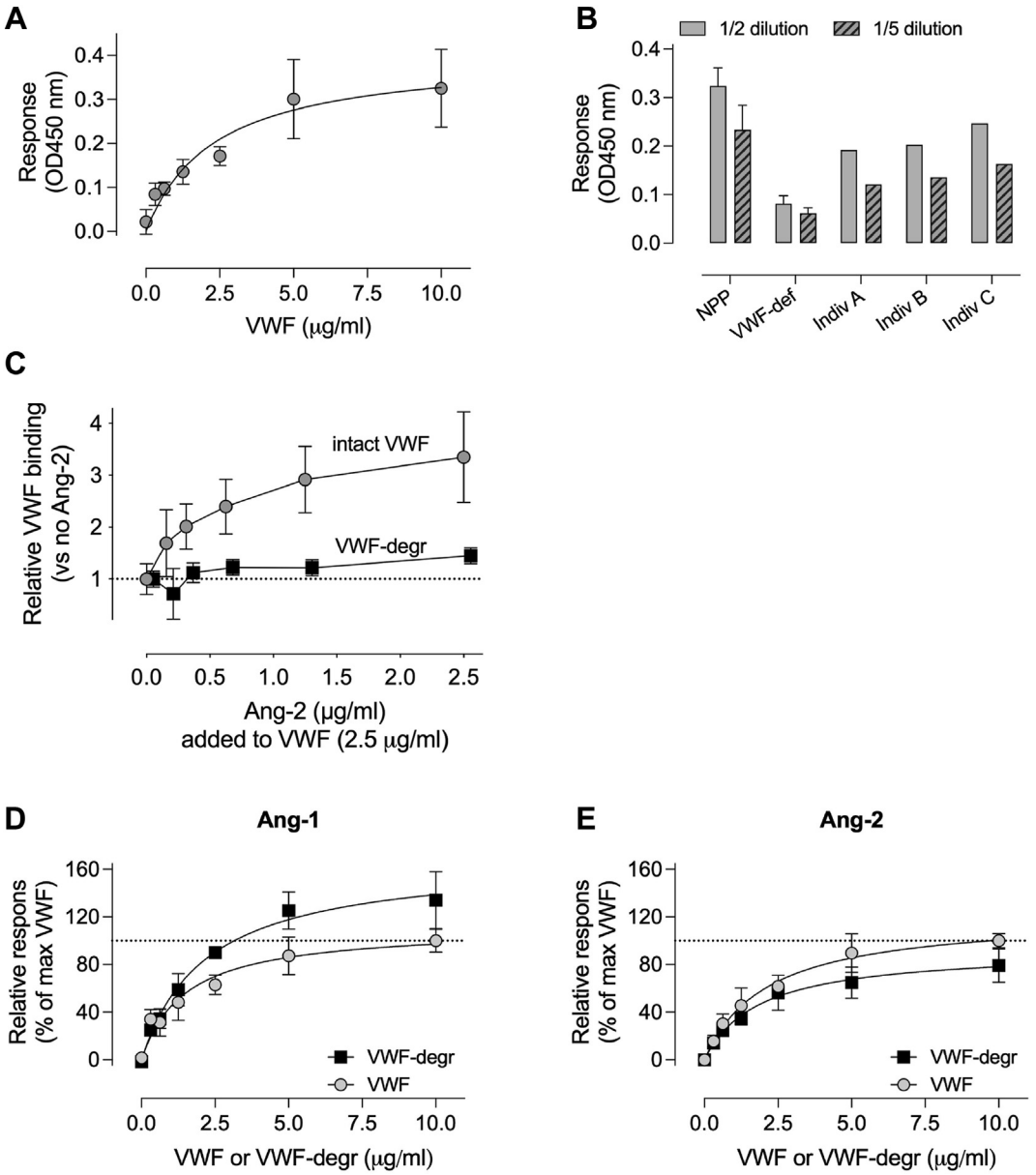


FIGURE 6 VWF interacts with Ang-1.(A) VWF (0-10 μg/mL) was incubated with immobilized Ang-1 (1 μg/mL) in 20 mM Hepes (pH 7.0), 100 mM NaCl, 2.5 mM $CaCl_2$, 0.1 % BSA, 0.1% Tween-20. Data represent the mean ± SD of 7 experiments. (B) Microtiter wells coated with anti-Ang-1 antibodies were incubated with diluted plasma (gray bars: 2-times diluted; hatched bars: 5-times diluted). Bound complex was then probed using peroxidase-labeled polyclonal anti-VWF antibodies and detected via hydrolysis of 3,3',5,5'-tetramethydlbenzidine. Data represent mean values of 3 experiments ± SD for NPP and VWF-deficient plasma, and the mean of a duplicate measurement for the individual plasmas. (C) Intact VWF (2.5 μg/mL) or degraded VWF was preincubated with Ang-2 (0-12.5 μg/mL) in 20 mM Bis-Tris buffer pH 6.8, 10 mM $CaCl_2$, 0.1% Tween-20, 0.03% bovine serum albumin. Subsequently, preparations were diluted 5-fold into 20 mM Hepes (pH 7.0), 100 mM NaCl, 2.5 mM $CaCl_2$, 0.1 % bovine serum albumin, 0.1% Tween-20 before addition to wells coated with Ang-1 (1 μg/mL). Bound VWF was then probed using peroxidase-labeled polyclonal anti-VWF antibodies and detected via hydrolysis of 3,3',5,5'-tetramethylbenzidine. (D, E) Binding of intact VWF and degraded VWF to immobilized Ang-1 and Ang-2 was performed as described in the legends of Figures 2A and 6A. Plotted is relative binding compared with 10 μg/mL intact VWF vs concentration of intact VWF or ADAMTS-13-degraded VWF (VWF-degr). Data represent mean ± SD of 4 experiments. NPP, normal pooled plasma; VWF, von Willebrand factor; VWF-def, von Willebrand factor-deficient plasma; Indiv A-C: plasma of individuals A, B, and C.

FVIII could affect binding of VWF to Ang-2. Indeed, increasing concentrations of FVIII (up to 30 μg/mL) dose-dependently reduced binding of VWF to Ang-2 by a maximum of 77% (Figure 5C). We conclude that Ang-2 will have little effect on binding of VWF to ADAMTS-13, whereas its interactive site in the D'D3-region may overlap with that of FVIII.

3.7 | VWF also binds to Ang-1

In view of the structural homology between Ang-2 and Ang-1, we explored the option that VWF is also able to bind Ang-1. Since Ang-1 is not located in the endothelial WPBs, binding was assessed under plasma-like conditions (high salt, low CaCl2, neutral pH).

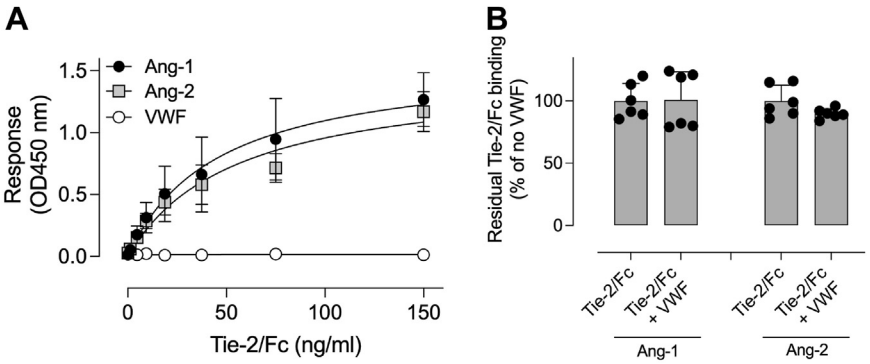


FIGURE 7 Effect of VWF degradation on binding to Ang-1 and Ang-2.(A) Tie-2/Fc (0-150 ng/mL) was incubated with immobilized Ang-1 (black circles), Ang-2 (gray squares), or VWF (white circles) in 20 mM Hepes (pH 7.0), 100 mM NaCl, 2.5 mM $CaCl_2$, 0.1 % BSA, 0.1% Tween-20. Bound Tie-2/Fc was probed with peroxidase-labeled anti-Fc antibodies and detected via hydrolysis of 3,3',5,5'-tetramethylbenzidine. (B) Immobilized Ang-1 or Ang-2 were incubated with VWF (20 μg/mL) for 1 hour. Wells were emptied and then incubated for 1 hour with Tie-2/Fc (50 ng/mL) and VWF (20 ug/mL). Bound Tie-2/Fc was probed with peroxidase-labeled anti-Fc antibodies and detected via hydrolysis of 3,3',5,5'-tetramethylbenzidine. Data represent mean ± SD of 6 measurements. Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; VWF, von Willebrand factor.

Unexpectedly, VWF bound in a dose-dependent manner to immobilized Ang-1, with half-maximal binding being obtained at 1.8 ± 1.0 μg/mL (Figure 6A). Since this concentration is below the average VWF plasma concentration (10 μg/mL), it would predict that VWF/Ang-1 complexes could potentially circulate in plasma. We therefore used a similar immunosorbent assay approach as for Ang-2. Wells coated with anti-Ang-1 antibodies were incubated with plasma and bound complexes were probed using anti-VWF antibodies. Complexes were readily detected in NPP but were absent in VWF-deficient plasma (Figure 6B). Complexes were also detected in the plasma of 3 individuals (Figure 6B). We then investigated whether Ang-1 and Ang-2 occupy overlapping binding sites within VWF. Therefore, binding of VWF to immobilized Ang-1 was tested in the presence of increasing concentrations of Ang-2. Surprisingly, binding of VWF to Ang-1 was stimulated by the presence of Ang-2 rather than being inhibited (Figure 6C). OD-values for VWF binding were increased up to 3.3 ± 0.9-fold ($P < .0001$) in the presence of Ang-2 at concentrations between 0.15 and 2.5 μg/mL of Ang-2. This suggests that binding of Ang-1 to VWF is modulated by the presence of Ang-2, and points to Ang-1 and Ang-2 occupying different binding sites within VWF.

3.8 | Binding of Ang-1 and Ang-2 to degraded VWF

Knowing that increased VWF degradation (eg, in VWD-type 2A) is associated with increased risks of angiodysplasia [27], we tested if binding of Ang-1 and Ang-2 to VWF is modified upon its degradation by ADAMTS-13. We therefore examined the binding of plasma-derived VWF (referred to as intact VWF) and degraded VWF (referred to as VWF-degr, ie, >85% proteolyzed by recombinant ADAMTS-13) [19] to immobilized Ang-1 or Ang-2. With maximal binding for intact VWF being arbitrarily set at 100%, it appeared that VWF-degr displayed slightly enhanced binding to Ang-1 (Bmax 151% (95% CI: 133%-176%) vs 100%; $P = .0008$; Figure 6D). In contrast, VWF-degr was somewhat less efficient in binding to immobilized Ang-2 (Bmax 76% (95% CI: 65%-92%) vs 100%; $P = .0121$; Figure 6E). These opposite effects prompted us to evaluate the effect of Ang-2 on binding of VWF-degr to Ang-1. Whereas Ang-2 stimulates binding of intact VWF to Ang-1, this capacity is lost when VWF is degraded by ADAMTS-13 (Figure 6C).

3.9 | Binding of Ang-1 and Ang-2 to Tie-2/Fc in the absence or presence of VWF

The cellular receptor for Ang-1 and Ang-2 on endothelial cells is Tie-2. We therefore investigated whether complex formation between these proteins is affect by VWF. In direct binding experiments, Tie-2/Fc bound efficiently to immobilized Ang-1 or Ang-2, whereas no binding of Tie-2/Fc to VWF was detected (Figure 7). Half-maximal binding was 0.2 ± 0.1 nM for the association of Tie-2 to both Ang-1 and Ang-2 (Table). Binding of Tie-2 to both Ang-1 and Ang-2 was unaffected by the presence of VWF (20 μg/mL).

4 | DISCUSSION

It is well-established that VWF is key to the formation of WPBs in endothelial cells, requiring the presence of both the VWF propeptide and the N-terminal portion of the mature VWF subunit [2,28,29]. It has been demonstrated that these organelles also include a variety of other proteins, and unbiased proteomic analysis revealed that >200 unique proteins are associated with these storage-organelles, including Ang-2 [30].

The presence of Ang-2 in WPBs was first reported in 2004, and its physiological relevance with regard to inflammation and angiopoiesis

TABLE Binding parameters for interactions between VWF or its derivatives with Ang-2 or Ang-1.

Immobilized protein	Protein in solution	Half-maximal binding		Figure
		µg/mL	nM	
Ang-2	VWF	0.5 ± 0.1	2.0 ± 0.4	2A
VWF	Ang-2	0.3 ± 0.1	5.3 ± 1.0	2B
Ang-2	A1-Fc	0.6 ± 0.1	6.1 ± 1.0	4A
Ang-2	A2-Fc	3.4 ± 0.9	36.7 ± 9.7	4A
Ang-2	A3-Fc	N.D.	N.D.	4A
Ang-2	D'D3-HPC4	8.9 ± 2.7	159 ± 48	4B
Ang-2	D4-Fc	2.3 ± 0.7	18.0 ± 6	4B
ADAMTS-13	VWF	0.3 ± 0.1	1.2 ± 0.4	5A
Ang-1	VWF	1.8 ± 1.0	7.2 ± 4.0	6A
Ang-1	Tie-2/Fc	0.05 ± 0.03	0.2 ± 0.1	7A
Ang-2	Tie-2/Fc	0.05 ± 0.03	0.2 ± 0.1	7A
VWF	Tie-2/Fc	N.D.	N.D.	7A

Ang-1, recombinant angiopoietin-1; Ang-2, recombinant angiopoietin-2; VWF, purified plasma-derived von Willebrand factor; N.D., binding curve did not allow for proper calculation of half-maximal binding.

has since been shown in several elegant studies [5,6,31,32]. Despite these insightful studies, the molecular basis of the potential interaction between VWF and Ang-2 remained unclear. Mobayen et al. [15] recently showed that optimal binding between Ang-2 and VWF requires conditions that are found in the cis-Golgi network, ie, low pH (pH 6.8), high $CaCl_2$ (10 mM), with no additional NaCl added (since increasing Cl^--ion concentration proved inhibitory to the interaction) [33]. In our studies, we could indeed confirm that these conditions favor interactions between VWF and Ang-2. In this regard, Ang-2 is similar to osteoprotegerin, which also binds optimally to VWF under these conditions [24]. It should be noted that when association has taken place under these conditions, change into plasma-like conditions (high salt, low $CaCl_2$, and neutral pH) did not result in a loss of complex between VWF and osteoprotegerin, and such complexes could be readily found in plasma [24]. Akin to the VWF/osteoprotegerin interaction, it now seems that also Ang-2 remains bound to VWF upon secretion into the circulation. First, Mobayen et al. [15] showed that the complex remains associated upon secretion from cultured endothelial cells. Second, we detected the presence of circulating VWF/Ang-2 complexes in plasma using immunoprecipitation and immunosorbent assays. Although we cannot completely exclude that some new complexes are being formed within the circulation, it seems more likely that such complexes are the remnants of those formed within the endothelial cells.

With regard to the binding efficiency between Ang-2 and VWF, we determined half-maximal binding between 2 and 5 nM. These are similar to the values found by Mobayen et al. [15] (3-5 nM). Both Mobayen et al. [15] and us identified the A1-domain as the dominant site of interaction, but we differed in the observation that we also identified lower affinity binding sites outside the A1-domain, in the D'D3-region, the A2- and D4 domains. Having identified multiple binding sites raises perhaps the question regarding the specificity of these interactions. However, with the exception of the D4-domain, binding of these VWF domains to Ang-2 was significantly reduced in the presence of domain-specific antibodies. Unexpectedly, binding of the VWF D'-D3 region to Ang-2 was inhibited by a monoclonal antibody known to interfere with FVIII binding. Indeed, FVIII also proved to inhibit in a dose-dependent manner binding of the VWF D'-D3 region to Ang-2, suggesting that binding sites for both proteins in the D'D3 region overlap at least to some extent. It should be noted that the recombinant VWF D'-D3 fragment used in this study was generated in the presence of the VWF propeptide, inducing natural dimerization of this fragment. In contrast, another variant in which dimerization was induced via the C-terminal side proved unable to bind to Ang-2 (data not shown). Apparently, propeptide-induced formation of inter-molecular disulfide bridges seems to be essential for correct exposure of the Ang-2 binding site. This would be compatible with previous reports showing that optimal FVIII binding requires propeptide-induced dimerization of VWF [34,35]. Of note, given the considerable higher affinity of FVIII for the D'D3-region (<1 nM), and the low concentrations of both FVIII and Ang-2 in the circulation (350 pM and 30 pM, respectively) compared with VWF (35 nM) [36,37], it is unconceivable that Ang-2 and FVIII will compete for VWF binding under physiological conditions. Indeed, no differences in Ang-2 levels were observed between plasma samples from normal controls and hemophilia A patients (Supplementary Figure S2).

One unexpected finding of our study was the capacity of VWF not only to interact with Ang-2 but also with Ang-1. Half-maximal binding of VWF was in the same range as found for Ang-2 (7.2 ± 4.0). Based on the maximal optical density (OD)-values that were obtained (OD = 0.3 for Ang-1 and OD = 0.9 for Ang-2), it seems that fewer binding sites are available for Ang-1. How and where VWF and Ang-1 will meet is uncertain at this point, but since Ang-1 is not present in WPBs, it is possible that complex formation most likely occurs in the circulation. A second surprising finding in this regard was that Ang-1 and Ang-2 do not compete for the same binding sites. In contrast, it appears that the presence of Ang-2 stimulates binding of VWF to Ang-1. We noticed that Ang-1 and Ang-2 did not bind to each other, suggesting that enhanced binding is not caused by such interactions. It cannot be excluded that Ang-2 induces subtle changes in the VWF conformation, thereby promoting Ang-1 binding. Evidently, additional studies are needed to define the mechanism by which Ang-2 promotes binding of VWF to Ang-1.

Since Ang-1 and Ang-2 do not bind to the same site on VWF, the possibility exists that different regions from Ang-1 and Ang-2 are involved in the interaction with VWF. Both Ang-1 and Ang-2 consist of an N-terminal coiled-coil domain, a short-linker peptide region and a C-terminal fibrinogen homology domain, which is involved in Tie-2 binding [16]. Binding of Ang-2 to VWF leaves Tie-2 binding unaffected [15], suggesting that the VWF binding site is outside the fibrinogen homology domain. Whether this is also true for Ang-1 remains to be determined.

Having identified the capacity of VWF to interact with both Ang-1 and Ang-2, the questions that follows is of course what this would mean from a physiological or pathologic point of view. As for the possibility of VWF to play a role in maintaining plasma levels of Ang-1 and/or Ang-2, it has been shown that Ang-2 and Ang-1 levels are similar between the various VWD-subtypes (VWD-type 1, VWD-type 2, and VWD-type 3) [38]. In addition, Ang-2 levels were indistinguishable between wild-type and VWF-knock out mice [39]. Thus, VWF is not required to maintain plasma levels of Ang-1 and Ang-2. With regard to the function of Ang-2, Schillemans et al. [13,40] recently showed that in the VWF-knock out endothelial cells, an increased associated of Ang-2 with its receptor Tie-2 was observed, which would be compatible with the more angiogenesis-prone condition observed in VWF-knock out mice [13,40]. In addition, Mobayen et al. [15] showed that complex formation with VWF does not prevent Ang-2 from binding to Tie-2. We here showed that VWF indeed is unable to prevent binding of Ang-1 or Ang-2 to Tie-2 (Figure 7). Thus, VWF seems to play a minor role in regulating Ang-2/Tie-2 interactions once Ang-2 is in the circulation. In view of our finding that Ang-2 promotes binding of Ang-1 to VWF, it is tempting to speculate that by doing so, the role of VWF-bound Ang-2 is to reduce levels of free Ang-1 in the circulation. In contrast to Ang-2, Ang-1 is known to bind to the extracellular matrix [16]. It is possible that by promoting VWF/Ang-1 complex formation, Ang-2 uses this approach to reduce extravascularization of Ang-1. By doing so, it could potentially prevent Ang-1 from using the extravascular space to interact with Tie-2.

ACKNOWLEDGMENTS

We thank Dr S. Shahbazi and Mrs P. Legendre for technical assistance in the early stages of the project. This work received financial support from the National Research Agency (Agence Nationale de la Recherche, ANR-20-CE19-0032-FLAMRING).

FUNDING

This work received financial support from the National Research Agency (Agence Nationale de la Recherche, ANR-20-CE19-0032-FLAMRING).

AUTHOR CONTRIBUTIONS

A.T. and O.D.C. performed experiments; A.T., P.J.L., C.V.D., S.R., and O.D.C. analyzed data; P.J.L., C.V.D., S.R., and O.D.C. designed and supervised the study; O.D.C. and P.J.L. wrote the manuscript and all authors were involved in the editing of the manuscript.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.rpth.2023.102204